Anoxybacter fermentans gen. nov., sp. nov., a piezophilic, thermophilic, anaerobic, fermentative bacterium isolated from a deep-sea hydrothermal vent

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A novel piezophilic, thermophilic, anaerobic, fermentative bacterial strain, designated strain DY22613T, was isolated from a deep-sea hydrothermal sulfide deposit at the East Pacific Rise (GPS position: 102.6°W 3.1°S). Cells of strain DY22613T were long, motile rods (10 to 20 μm in length and 0.5 μm in width) with peritrichous flagella and were Gram-stain-negative. Growth was recorded at 44–72 °C (optimum 60–62 °C) and at hydrostatic pressures of 0.1–55 MPa (optimum 20 MPa). The pH range for growth was from pH 5.0 to 9.0 with an optimum at pH 7.0. Growth was observed in the presence of 1 to 8 % (w/v) sea salts and 0.65 to 5.2 % (w/v) NaCl, with optimum salt concentrations at 3.5 % for sea salts and at 2.3 % for NaCl. Under optimal growth conditions, the shortest generation time observed was 27 min (60 °C, 20 MPa). Strain DY22613T was heterotrophic, able to utilize complex organic compounds, amino acids, sugars and organic acids including peptone, tryptone, beef extract, yeast extract, alanine, glutamine, methionine, phenylalanine, serine, threonine, fructose, fucose, galactose, gentiobiose, glucose, mannose, melibiose, palatinose, rhamnose, turanose, pyruvate, lactic acid, methyl ester, erythritol, galacturonic acid and glucosaminic acid. Strain DY22613T was able to reduce Fe(III) compounds, including Fe(III) oxyhydroxide (pH 7.0), amorphous iron(III) oxide (pH 9.0), goethite (α-FeOOH, pH 12.0), Fe(III) citrate and elemental sulfur. Products of fermentation were butyrate, acetate and hydrogen. Main cellular fatty acids were iso-C15 : 0, iso-C14 : 0 3-OH and C14 : 0. The genomic DNA G+C content of strain DY22613T was 36.7 mol%. Based on 16S rRNA gene sequence analysis, the strain forms a novel lineage within the class Clostridia and clusters with the order Haloanaerobiales (86.92 % 16S rRNA gene sequence similarity). The phylogenetic data suggest that the lineage represents at least a novel genus and species, for which the name Anoxybacter fermentans gen. nov., sp. nov. is proposed. The type strain is DY22613T (=JCM 19466T=DSM 28033T=MCCC 1A06456T).

Abbreviations: AODS, 9,10-anthraquinone-2,6-disulfonate; S*, elemental sulphur.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of strain DY22613T is KC794015.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.
Molecular inventories revealed a wide diversity of thermophilic prokaryotes at deep-sea hydrothermal vents, only some of which have been cultivated (Miroshnichenko & Bonch-Osmolovsaya, 2006). Some thermophiles of the class Clostridia have been isolated from deep-sea vents, including representatives of the genera Caloranaerobacter (Wery et al., 2001), Caminicella (Alain et al., 2002), Tepidibacter (Slobodkin et al., 2003; Urios et al., 2004), Caldanaerobacter (Fardeau et al., 2004) and Clostridium. Two species of the genus Clostridium have also been isolated from deep-sea vents, namely, Clostridium caminithermale (Brisbarre et al., 2003) and Clostridium tepidiprofundii (Slobodkina et al., 2008). Recently, one novel species, Vallitalea pronyensis, has been isolated from a shallow hydrothermal vent chimney (Ben Aissa et al., 2014). These six genera fall into the class Clostridia, a highly polyphyletic class of obligate anaerobes. Most of them ferment carbohydrates to acetate, ethanol, H₂ and CO₂. At the time of writing, the class Clostridia encompasses four orders including Clostridiales, Halanaerobiales, Natranaerobiales and Thermoanaerobacterales and the sub-order EuBacteriineae (Rainey, 2009). The orders Halanaerobiales and Natranaerobiales were created to accommodate halophilic anaerobes (Rainey et al., 1995; Mesbah et al., 2007). The order Thermoanaerobacterales is polyphyletic, encompassing species able to survive in environments of extreme elevated temperature (Hogan, 2010). The order Clostridiales is highly polyphyletic, not a natural group, with diverse clades. We describe in this report the characterization of a novel piezophilic, anaerobic, thermophilic, diverse clades. We describe in this report the characterization of a novel piezophilic, anaerobic, thermophilic, highly polyphyletic class of obligate anaerobes. Most of them ferment carbohydrates to acetate, ethanol, H₂ and CO₂. At the time of writing, the class Clostridia encompasses four orders including Clostridiales, Halanaerobiales, Natranaerobiales and Thermoanaerobacterales and the sub-order EuBacteriineae (Rainey, 2009). The orders Halanaerobiales and Natranaerobiales were created to accommodate halophilic anaerobes (Rainey et al., 1995; Mesbah et al., 2007). The order Thermoanaerobacterales is polyphyletic, encompassing species able to survive in environments of extreme elevated temperature (Hogan, 2010). The order Clostridiales is highly polyphyletic, not a natural group, with diverse clades. We describe in this report the characterization of a novel piezophilic, anaerobic, thermophilic, fermentative bacterium (designated strain DY22613T) isolated from a deep-sea hydrothermal vent environment. On the basis of the physiological and phylogenetic evidence presented, we propose a novel genus, Anoxybacter gen. nov., to accommodate this micro-organism.

Strain DY22613T was isolated from hydrothermal sulfides collected in July 2011 at a depth of 2891 m at the East Pacific Rise (GPS position: 102.6°W 3.1°S), during the DY125-22 cruise of RV Da Yang Yi Hao. Sulfide samples were collected using a benthic seabed grab and stored hermetically in sealed sterile vials. Samples were transported at 4°C to the laboratory. A sample composed of hydrothermal chimney fragments bearing polychaete tubes and tube worms was chosen to perform enrichment cultures of thermophilic heterotrophic anaerobes. X-ray diffraction analysis indicated that this sample was mainly composed of pyrite (FeS₂) and sphalerite (ZnS).

One subsample was used to inoculate (1/10, w/v) a sterile liquid medium called FRPFO, which was prepared anaerobically and kept under an atmosphere of highly purified 100% nitrogen. FRPFO medium contained (g l⁻¹, unless stated otherwise): peptone (10), sea salts (30; Sigma), PIPES (6.05), cysteine hydrochloride (0.5), resazurin (1 mg) and amorphous Fe(III) oxyhydroxide (50 mM, pH 7.0) as an electron acceptor. Enrichment cultures were incubated at 60°C. Between 3 to 5 days of incubation, the colour of the precipitates changed from brown to black, indicating Fe(III) reduction. The enriched microbial community was composed of motile long and small rods. One strain, designated DY22613T, was unable to form colonies in solidified medium containing 1.5% (w/v) agar or 0.2% (w/v) Gelrite, therefore, strain DY22613T was purified by three repeated dilution-to-extinction series. The purity of this isolate was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using several PCR primers (Lane, 1991) (Bac8F, Bac27F, 1100R, U1492R). Stock cultures were stored at −80°C in FRPFO medium supplemented with 5% (v/v) DMSO.

The morphological characteristics of cells of the novel isolate were determined by using light microscopy (CX21; Olympus) and transmission electron microscopy (JEM-1230; JEOL). For ultrathin section examination of the cell wall, bacterial cells were fixed with osmic acid and embedded in araldite; the samples were then sliced and stained with lead citrate (Reynolds, 1963). Cells of strain DY22613T were regular to long rods (10 to 20 μm in length and 0.5 μm in width), motile, bearing flagella (Fig. S1a, available in the online Supplementary Material). Cells occurred mainly singly or formed short chains. The cells stained Gram-negative (Hangzhou Tianhe Micro-organism Reagent), and electron microscopy of ultrathin sections of cells revealed the presence of two layers characteristic of Gram-stain-negative bacteria (Fig. S1b). Moreover, the KOH reaction was positive, confirming the Gram-stain-negative type of the cells. Spores were not observed.

Physiological characterization of the novel isolate was carried out in FRPFO medium dispensed anaerobically in 50 ml vials sealed with butyl-rubber stoppers, reduced with 0.05% (w/v) cysteine hydrochloride sterile solution, just before inoculation. Unless stated otherwise, experiments were carried out anaerobically under an atmosphere of N₂ (100%, 1 bar) and incubations were performed in the dark at 60°C and pH 7.0. Growth was routinely monitored by direct cell counting using a modified Thomas chamber (depth 10 μm). Growth rates were calculated using linear regression analysis of eight points along the linear portions of the growth curves that were exponentially transformed. The determination of the temperature range for growth was tested over the range 40–74°C at 2°C intervals. Growth was observed from 44 to 72°C, with an optimum growth rate at 60–62°C. Growth was also observed under high hydrostatic pressure, from 0.1 to 55 MPa (optimum 20 MPa; Fig. S2). The pH range for growth was tested from initial pH 4.0 to initial pH 10.0 at 60°C in basal medium buffered and adjusted to the required pH (initial pH at 20°C) with MES buffer (pH 4.0–6.0), PIPES buffer (pH 7.0–8.0), HEPES buffer (pH 8.0–9.0), AMPSO buffer (pH 9.0–10.0). Growth was observed from pH 5.0 to 9.0 and the optimum pH for growth was pH 7.0. Salt tolerance was tested at 60°C in FRPFO medium prepared with various concentrations of NaCl (0–10%, w/v, at 0.5% intervals) or various concentrations of sea salts (0–100 g l⁻¹ at 5 g l⁻¹ intervals). Strain DY22613T required salt and...
grown on amorphous iron(III) oxide (pH 9.0), Fe(III) citrate, Fe(III) oxyhydroxide (pH 7.0) and goethite (pH 12.0), respectively (experiments were performed in triplicate). The novel isolate reduced elemental sulfur (S\(^{2-}\)) to hydrogen sulfide (checked by colorimetry in the presence of CuSO\(_4\)/HCl), but did not reduce sulfate, sulfite, thiosulfate or nitrate.

The determination of the whole-cell fatty acid composition was performed on cultures grown on YT medium at 60 °C. Cells were harvested at the end of the exponential growth phase (20 h of incubation). Fatty acids were extracted and analysed following the instructions of the Sherlock Microbial Identification System (MIDI). The fatty acids in strain DY22613\(^{T}\) comprised iso-C\(_{15:0}\) (36.26 %), iso-C\(_{14:0}\) 3-OH (20.61 %) and C\(_{14:0}\) (7.36 %) and differed from the type strain Halothrombix orenii H168\(^{T}\) in the proportion of several fatty acids including iso-C\(_{15:0}\) (54.3 % in H168\(^{T}\), C\(_{16:0}\) (9.94 %), anteiso-C\(_{15:0}\) (9.79 %) and C\(_{14:0}\) (7.96 %) (Cayol et al., 1994). The fatty acid profiles of both species are given in Table S1.

The genomic DNA G + C content of strain DY22613\(^{T}\) was 36.7 mol% as determined by genome sequencing using Illumina GAIIx (Meiji Company, Shanghai). An almost complete 16S rRNA gene sequence (1471 nt) was determined by double strand DNA sequencing. The identification of phylogenetic neighbours was initially carried out by BLAST (Altschul et al., 1997) and MEGABLAST (Zhang et al., 2000) against the database of type strains with validly published prokaryotic names (Chun et al., 2007). A search of the most similar 16S RNA gene sequences was also done against the web-based EzTaxon-e Server (Kim et al., 2012).

The 16S RNA gene sequence of strain DY22613\(^{T}\) was found to be very distantly related to species in the orders Halanaerobiales, Natranaerobiales, Thermoanaerobacterales and Clostridiales in the phylum Firmicutes, with similarity below 87.0 %. The closest relative was H. orenii H168\(^{T}\) (Cayol et al., 1994), with 86.92 % 16S rRNA gene sequence similarity, followed by Natranaerobius trueperi JW/NM-WN-LH1\(^{T}\) (85.73 %) (Mesbah & Wiegel, 2009) and Moorella humiferrea 64-FGQT\(^{T}\) (85.63 %) (Nepomnyashchaya et al., 2012).

A phylogenetic tree of representative members in the class Clostridia was reconstructed from 16S rRNA gene sequences using 1239 homologous gene sequence positions (Fig. 1). Alignment of all sequences was performed using the software CLUSTAL_X (version 2.3) and the phylogenetic tree was reconstructed using the neighbour-joining method with the software MEGA (version 5.1). Bootstrap analysis was performed with 1000 replications to provide confidence estimates for the tree topology. Based on this analysis, strain DY22613\(^{T}\) clearly belongs to the class Clostridia, but is not affiliated closely with any of the described lineages. Its closest neighbours belong to the order Halanaerobiales but are distantly related to the novel isolate (Fig. 1). Furthermore, strain DY22613\(^{T}\) could be differentiated from its closest relatives H. orenii, N. trueperi and M. humiferrea.
**Fig. 1.** Phylogenetic dendrogram obtained by neighbour-joining analysis based on 16S rRNA gene sequences (1239 bp, omitting unaligned regions), showing the position of strain DY22613ᵀ within the class *Clostridia*. Bar, expected number of changes per sequence position.

**Table 1.** Differential characteristics between strain DY22613ᵀ and its phylogenetically closest relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geographical origin</td>
<td>Sulfide of deep-sea hydrothermal vent, East Pacific rise</td>
<td>Sediment of salted lake, Tunisia</td>
<td>Sediment of alkaline, hypersaline lake, Egypt</td>
<td>Sediment of terrestrial hydrothermal spring, Russia</td>
</tr>
<tr>
<td>Cell size (µm)</td>
<td>10.0–20.0 × 0.5</td>
<td>10.0–20.0 × 0.4–0.6</td>
<td>4.0–20.0 (10)</td>
<td>2.0–5.0 × 0.3–0.5</td>
</tr>
<tr>
<td>NaCl range, %, w/v (optimum)</td>
<td>0.65–5.20 (2.3)</td>
<td>4.0–20.0 (10)</td>
<td>9.9–21.6 (13.4)</td>
<td>0–15.0 (0)</td>
</tr>
<tr>
<td>Temp. range, °C (optimum)</td>
<td>45.0–70.0 (60.0–62.0)</td>
<td>45.0–68.0 (60.0)</td>
<td>26.0–56.0 (51.0)</td>
<td>46.0–70.0 (65.0)</td>
</tr>
<tr>
<td>pH range (optimum)</td>
<td>5.0–9.0 (7.0)</td>
<td>5.5–8.2 (6.5–7.0)</td>
<td>8.5–11.5 (9.9)</td>
<td>5.5–8.5 (7.0)</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>36.7</td>
<td>39.6</td>
<td>41.7</td>
<td>51.0</td>
</tr>
<tr>
<td>Major fatty acids (&gt;7 %)</td>
<td>iso-C₁₅:₀ (36.26 %); iso-C₁₄:₀ 3-OH (20.61 %); C₁₄:₀ (7.36 %)</td>
<td>iso-C₁₅:₀ (54.30 %); C₁₆:₀ (9.94 %); anteiso-C₁₅:₀ (9.79 %); C₁₄:₀ (7.96 %)</td>
<td>iso-C₁₅:₀ (80.40 %); anteiso-C₁₅:₀ (9.40 %)</td>
<td>ND</td>
</tr>
<tr>
<td>16S rRNA gene sequence similarity (%)</td>
<td>100.00</td>
<td>86.92</td>
<td>85.73</td>
<td>85.63</td>
</tr>
</tbody>
</table>

*Calculated in reference to the 16S rRNA gene sequence of strain DY22613ᵀ.
based on a number of physiological characteristics such as NaCl range for growth and optimal salinity for growth, genomic DNA G+C content (mol%) and fatty acid profile (Table 1).

In conclusion, on the basis of the wide phylogenetic distance from its closest relatives (far below the threshold level of 94.5% identity for the delineation of a new genus and close to the threshold level of 86.5% for a new family level of 94.5% identity for the delineation of a new genus distance from its closest relatives (far below the threshold level of 86.5% for a new family level of 94.5% identity for the delineation of a new genus distance from its closest relatives), we propose to place strain DY22613T as the type strain of a novel species within the genus Anoxybac ter. Description of Anoxybac ter gen. nov.

Anoxybac ter (An.ox.y bac’ter. Gr. pref. an without; M.L. oxy shortened from oxygenium oxygen; N.L. bac ter masc. equivalent of Gr. neut. n. bakterion rod or sta; N.L. masc. n. Anoxybac ter rod growing without oxygen).

Cells are Gram-stain-negative. Endospores are not observed. Thermophilic. Strictly anaerobic. Chemolysogoheterotrophic. The genomic DNA G+C content is approximately 37 mol%.

Description of Anoxybac ter fermentans sp. nov.

Anoxybac ter fermentans (fer.men’tans. L. part. adj. fer mentans fermenting).

Cells are motile, round-ended rods with flagella. Cells grow in the temperature range of 44 to 72 °C (optimum 60–62 °C), in the hydrostatic pressure ranging from 0.1 to 55 MPa (optimum 20 MPa), pH range of 5.0 to 9.0 (optimum pH 7.0) and with 10 to 85 g l⁻¹ sea salts (optimum 35 g l⁻¹). The shortest doubling time is 27 min at 60 °C under 20 MPa. It can utilize complex organic compounds, amino acids, and organic acids including peptone, tryptone, beef extract, yeast extract, alanine, glutamine, methionine, phenylalanine, serine, threonine, fructose, fucose, galactose, gentiobiose, mannose, melibiose, glucose, palatinose, rhamose, rhamnose, turanose, pyruvate, lactate, galacturonic acid, glucosamine acid, methly ester and erythritol. Insoluble Fe(III) compounds, including amorphous Fe(III) oxyhydroxide (pH 7.0), amorphous iron (III) oxide (pH 9.0), goethite (x-FeOOH; pH 12.0) and Fe(III) citrate can be reduced to Fe(II). Reduces S² but does not reduce sulfite, sulfate, thiosulfate or sulfate.

The type strain, DY22613T (=JCM 19466T=DSM 28033T=MCCC 1A06456T), was isolated from a hydrothermal sulfide sample collected from an East Pacific Ocean hydrothermal field (GPS position: 102.6° W 3.1° S) at a depth of 2891 m. The genomic DNA G+C content of the type strain is 36.70 mol%.

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